Reference-Free Thio-Succinimide Isomerization Characterization by Electron-Activated Dissociation

Junyan Yang[1], Jiaqi Yuan[1], Yue Huang[1], Anton I. Rosenbaum[1*]

[1]Integrated Bioanalysis, Clinical Pharmacology & Safety Sciences, R&D, AstraZeneca,121 Oyster Point Blvd., South San Francisco, CA 94080, USA

*Correspondence

Anton I. Rosenbaum - 121 Oyster Point Blvd, South San Francisco, CA 94080, USA; Tel: +1-650-379-3099; E-mail: anton.rosenbaum@astrazeneca.com

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Abstract

Rationale

Isomerism can be an important aspect in pharmaceutical drug development. Identification of isomers can provide insights into drug pharmacology and contribute to better design of drug molecules. The general approaches to differentiate isomers include Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), and circular dichroism. Although proven effective, a commonly used method to differentiate isomers is chromatography coupled with mass spectrometry (MS). Notably, MS is routinely applied in leucine and isoleucine differentiation to facilitate protein sequencing. This work focuses on isomer differentiation of widely applied thio-succinimide structure bridging the antibody backbone and linker-payload of antibody-drug conjugates (ADCs). The hydrolysis of thio-succinimide stabilizes the payload-protein structure while generating a pair of constitutional isomers: thio-aspartyl and thio-isoaspartyl.

Methods

This paper introduces a hybrid method using ligand binding assay (LBA) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to reveal isomerization details of thio-succinimide hydrolysis over time in plasma samples incubated with ADC. By applying two orthogonal fragmentation methods, collision-induced dissociation (CID) and electron-activated dissociation (EAD), this pair of isomers showed different MS/MS spectra. This observation enables a unique approach in distinguishing thio-succinimide hydrolysis isomers.

Results

We observed signature R_1+Thio+57 , R_2+Succ+H_2O-57 and R_2+Succ+H_2O-44 (Succ = succinate) fragment ions that differentiated thio-aspartyl and thio-isoaspartyl isomers using EAD. A newly discovered R_2+ThioSucc+H_2O-44 ion also served as additional evidence that further supported our findings.

Conclusions

This study is a first-to-date identification of thio-succinimide hydrolysis isomers without using synthesized reference materials. This approach should be applicable to all thio-succinimide-linked molecules. Correct identification of thio-succinimide hydrolysis isomers may eventually benefit the development of ADCs in the future.

1. Introduction

Thiol-maleimide conjugation is a highly popular reaction due to its specificity, selectivity and fast kinetics.[1] In recent decades, this reaction has been used in various fields, including biomolecule labelling[2] and synthesis of novel polymeric materials.[3] One of the most important applications of the thiol-maleimide reaction is the bioconjugation between linker-payload and antibody backbone, to generate antibody-drug conjugates (ADCs).[4, 5] Comparing to well-developed small molecule drugs, ADC shows promise as a new class of therapeutics, with the first ADC approved in 2001. As of October 2023, there are 15 different ADCs approved, and 10 of them utilize thiol-maleimide reaction during their syntheses.[6]

There are mainly two competitive biotransformation processes occurring at the thio-succinimide site from linker. The reversible Retro-Michael reaction of thio-succinimide leads to the pre-mature linker-payload deconjugation.[7] Different approaches have been tested to avoid such problem.[8-10] On the other hand, the thio-succinimide is also subject to ring opening hydrolysis, and its product prevents the deconjugation, increasing the stability of ADCs.[7] Notably, the hydrolysis of thio-succinimide results in a pair of isomeric products, thio-aspartyl (thio-Asp) and thio-isoaspartyl (thio-isoAsp).[11] This finding was confirmed by observations from several analytical techniques, including FTIR and NMR.[10] Nevertheless, to our knowledge, the differentiation of these isomers using LC-MS has never been reported.

Biotransformation of ADCs is predominantly characterized by intact protein mass analysis through liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS).[12] Previously, we reported a workflow that uses hybrid ligand-binding assay (LBA) coupled with intact protein mass LC-HRMS to study ADC drug-antibody ratio (DAR) changes resulted from biotransformation.[13] However, the isomeric structures cannot be distinguished through the intact protein mass analysis due to their exact same mass. Furthermore, the reverse phase LC (RPLC) may not be sufficient to separate large molecules with such subtle structural difference.[12] Other than intact mass analysis, researchers also synthesize reference materials and compare their retention time (RT) to identify isomers.[14] The complicated synthesis and purification processes compromise the efficiency of this analytical approach. Here, we report a bottom-up LC-MS/MS approach with the help from newly developed electron-activated dissociation (EAD) fragmentation to distinguish thio-succinimide hydrolysis isomeric products.

Electron-based dissociation (ExD) fragmentation methods have a long history in aiding the characterization of biomolecules.[15-17] The electron-capture dissociation (ECD) method primarily cleaves peptide backbone,[18] resulting in c and z ions,[19] thus obtaining a higher sequence coverage compared to the traditional collision-induced dissociation (CID) fragmentation.[20,21] Besides, ECD leaves the side chain intact, making this fragmentation method useful in

identifying post-translational modifications of proteins.[22] Hot ECD (hECD), which employs higher kinetic energy, enables secondary fragmentation, which can, for instance, differentiate leucine and isoleucine.[23] However, conventional ExD fragmentation methods, such as ECD and electron-transfer dissociation (ETD), mainly fragment multiply charged ions,[20] leaving singly or doubly charged ions less accessible to fragmentation. Recently, EAD, which comprises of ECD and hECD, was shown to be flexible in fragmenting ions with a wide range of charge states.[24] The enzymatic digestion of ADCs yields peptides with various length and small molecule linker-payloads, making EAD highly valuable for the characterization of ADCs, where the fragmentation of differently protonated species may be achieved.

Thio-succinimide hydrolysis is analogous to protein deamidation. The asparagine deamidation generates a succinimide intermediate before the addition of water.[25] The resulting aspartic acid results in ~ 1 Da molecular weight increase compared to its original mass. Similar to thio-succinimide hydrolysis, the asparagine deamidation also generates a pair of isomeric products, aspartic acid, and iso-aspartic acid. We previously reported the identification of asparagine deamidation at the complementarity-determining regions of another ADC, MEDI7247, employing LC-MS/MS.[26] The diagnostic ions were c+57 and z-57 coming from the iso-aspartic acid after fragmentation between α- and β-carbons through EAD.[27] Here, we investigated the feasibility of employing similar strategy (+57 u or -57 u related signature ions) to differentiate thio-Asp and thio-isoAsp, despite the slight structure difference compared to aspartic acid and iso-aspartic acid. Moreover, we further explored if EAD could generate more unique diagnostic ions only for thio-succinimide hydrolysis products. To our knowledge, we are the first to report an in-depth characterization of thio-succinimide hydrolysis isomerization using a bottom-up LC-MS/MS approach without requiring reference materials.

2. Methods

2.1 Materials

ADC1 and anti-idiotype (anti-ID) antibody were generated in-house by AstraZeneca (Gaithersburg, MD). The SMART IA magnetic beads, EZ-LINK Sulfo-NHS-LC-Biotin biotinylation kit, Zeba desalting spin columns, LC-MS grade formic acid (FA) and tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.5) were acquired from ThermoFisher Scientific (Waltham, MA). The Acquity UPLC BEH C18 columns and Oasis HLB solid phase extraction 96-well plates were purchased from Waters (Milford, MA). LC-MS grade acetonitrile (ACN), water, methanol and bovine serum albumin (BSA) were acquired from Sigma Aldrich (St. Louis, MO). Pooled human and CD1 mouse plasma were acquired from BioIVT (Hicksville, NY). Eppendorf Protein LoBind tubes, Deepwell 96-well plates, Lonza deionized (DI) water and all other general reagents and supplies were purchased from VWR Scientific (Radnor, PA).

2.2 Instrumentation

LC-MS/MS experiments were performed on ZenoTOF 7600 mass spectrometer (SCIEX, Toronto), coupled with Exion UHPLC system. The mass spectra were analyzed using a research version PeakView (version number: 1.2.2.0).

2.3 ADC1 incubation, immuno-affinity enrichment and sample clean-up

Figure S1 shows a brief description of experiment steps of sample preparation for analyzing thio-succinimide hydrolysis isomers. 20 mg/mL ADC1 stock solution was diluted to 1 mg/mL working solution using DI water. 2250 µL of human or CD1 mouse plasma was added into a 5 mL Protein LoBind tube containing 250 µL 1 mg/mL ADC1 working solution. The solution was briefly vortexed, before being split into 300 µL aliquots. The final concentration of ADC1 was 0.1 mg/mL. Three aliquots were incubated at 37°C for 168 h using digital HeatBlock (VWR) then stored at -80°C until use, whereas three other aliquots were immediately stored at -80°C after solution preparation (denoted as 0 h).

The biotinylation of anti-ID antibody was performed using an EZ-LINK biotinylation kit following the manufacturer's protocol. The biotinylated anti-ID antibody was purified using a Zeba 7 kDa molecular weight cut-off desalting spin column, before being stored at -80°C. The final concentration of biotinylated anti-ID antibody was determined to be 8.48 mg/mL. To ensure sufficient capture of ADC1 in plasma incubated samples, the ratio between biotinylated anti-ID antibody and SMART IA magnetic beads was kept at 1:10 (the mass of biotinylated anti-ID antibody in µg to the volume of SMART IA magnetic beads in mL). The tube that contains the solution mixture was rotated for 30 minutes at room temperature using a LabQuake shaker (Barnstead), before loading onto a magnetic tube rack. SMART IA magnetic beads were separated from the solution, and the supernatant was extracted and discarded. The SMART IA beads were washed three times with SN1 buffer (50 mM Tris-HCl in DI water containing 1 mg/mL BSA, pH 7.5) with same volume as the original bead slurry. The anti-ID conjugated SMART IA beads were stored at 4°C until use.

1000 µl of SN1 buffer were added to a new 5 mL Protein LoBind tube, followed by mixing with 300 µL of plasma incubated ADC1 sample. The mixture was briefly vortexed before adding 600 µL conjugated SMART IA beads to the tube. The tube was taped onto a Thermomixer C (Eppendorf) and shaken at 25°C and 1200 rpm for 2 h. After immuno-affinity enrichment, the tube was put onto the magnetic tube rack, and supernatant was discarded. The beads were washed 3 times using 1900 µL of SN2 buffer (50 mM Tris-HCl in DI water, pH 7.5) before 1500 µL SMART IA digestion buffer (provided in the kit) was added to the washed beads. To activate the pre-immobilized trypsin on SMART IA beads, the mixture was shaken at 70°C and 1200 rpm using Thermomixer C (Eppendorf) for 2 h. After tryptic digestion, the tube was placed onto the magnetic tube rack, where digested ADC1 solution was transferred into a new tube. The sample clean-up was performed using an Oasis HLB cartridge (Waters) following the manufacturer's protocol. Post clean-up sample was diluted to 120 µL using 0.1% FA in water, and 30 µL sample was loaded to the LC-MS system.

2.4 LC-MS/MS parameters

The separation of tryptic digested peptides was performed on a BEH C18 UPLC column (Waters, 1.7 µm, 2.1×50 mm) using 0.1% FA in water as mobile phase A (MPA) and 0.1% FA in ACN as mobile phase B (MPB). The ZenoTOF 7600 mass spectrometer was operated in full-scan MS (m/z 100 to 1500) with collision energy (CE) set as 10 V. For MS/MS, CE = 40 V was

applied to CID fragmentation, whereas the kinetic energy (KE) for EAD fragmentation was set as 11 eV. The LC gradient and other parameters are described in the supporting information in detail (Section S2).

3. Results

3.1 Characterization of thio-succinimide hydrolysis

Thiol-maleimide reaction is widely used for bioconjugation purposes. The resulted thio-succinimide structure is vulnerable to ring-opening hydrolysis. ADC1 is a thio-succinimide-linked ADC, produced from reducing interchain disulfide bonds, followed by covalently conjugating linker-payload to the thiol groups from cysteine side chains using maleimide chemistry. To identify and characterize its hydrolysis isomers, we used a signature ADC1 tryptic digested product, which is composed of a tripeptide GEC, succinimide (with or without water adduct) and R_2 to demonstrate the process. Figure 1 shows the hydrolysis reaction, and potential diagnostic ions for differentiating hydrolysis isomers, using GEC+succinimide+R_2 as an example.

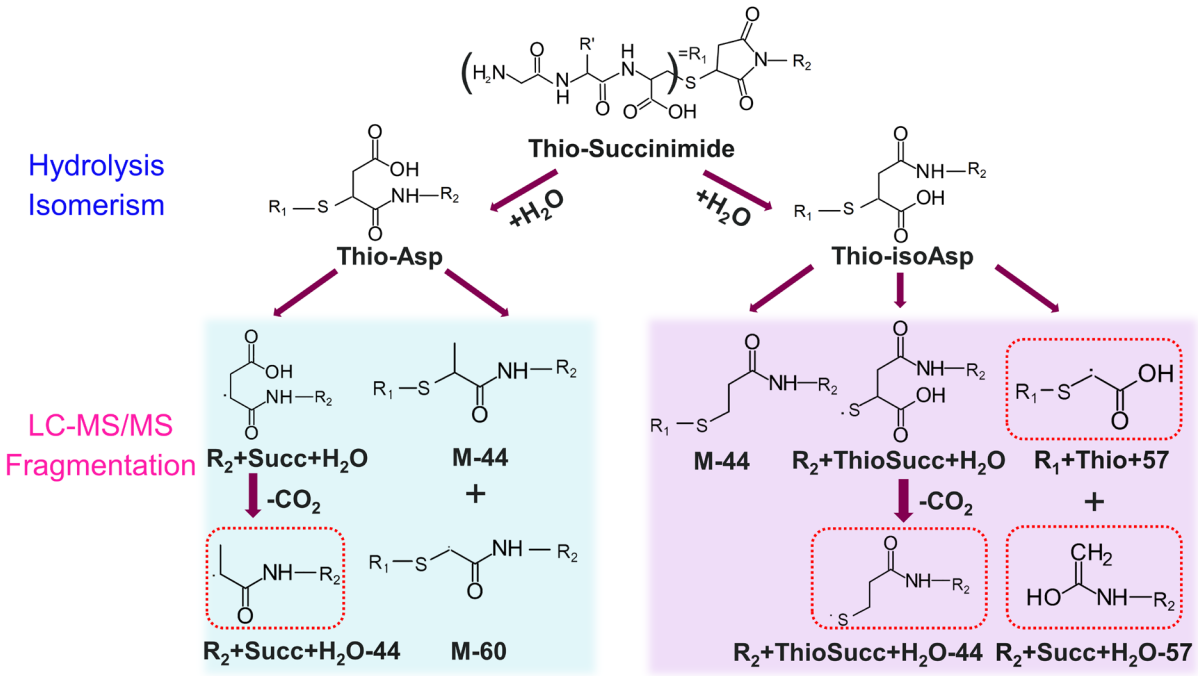

Figure 1. Scheme of thio-succinimide hydrolysis mechanism and the proposed structures of fragment ions after electron-activated dissociation (EAD) fragmentation. Fragment ions in the red boxes are diagnostic ions. R_1 is a tripeptide GEC, and R_2 is linker-payload. The linker-payload is covalently conjugated to the antibody *via* a cysteine side chain thiol. Succ+H_2O = hydrolyzed succinimide.

Figure 2A shows the mass spectra of GEC+succinimide+R_2 of ADC1 with 0 h (black line) and 168 h (blue line) incubation. After 168 h incubation, the signal intensity of GEC+succinimide+R_2 (observed monoisotopic *m/z* 485.8744, -11 ppm difference) dropped significantly compared to the sample with 0 h incubation. To be specific, the absolute intensity of the monoisotopic peak of GEC+succinimide+R_2 dropped from 2.9×10^4 to 7.2×10^2 cps after

168 h of incubation. Instead, we noticed a notable increase in peaks representing GEC+succinimide+R_2 with +18 Da shift (observed monoisotopic *m/z* 491.8779, -11 ppm difference). Thus, the hydrolysis of succinimide over 168 h was confirmed. Figure 2B is the extracted ion chromatogram (XIC) of GEC+succinimide+R_2 (*m/z* 485.8799) in 0 h and 168 h incubated sample. Figure 2C shows the XIC of GEC+succinimide+H_2O+R_2 (*m/z* 491.8834) at different incubation times. Similarly, the XICs support the conclusion that most of the succinimide conjugated to GEC is hydrolyzed over 168 hours of incubation. Table S2 in the supporting information gives theoretical and observed monoisotopic *m/z* values of discussed ions.

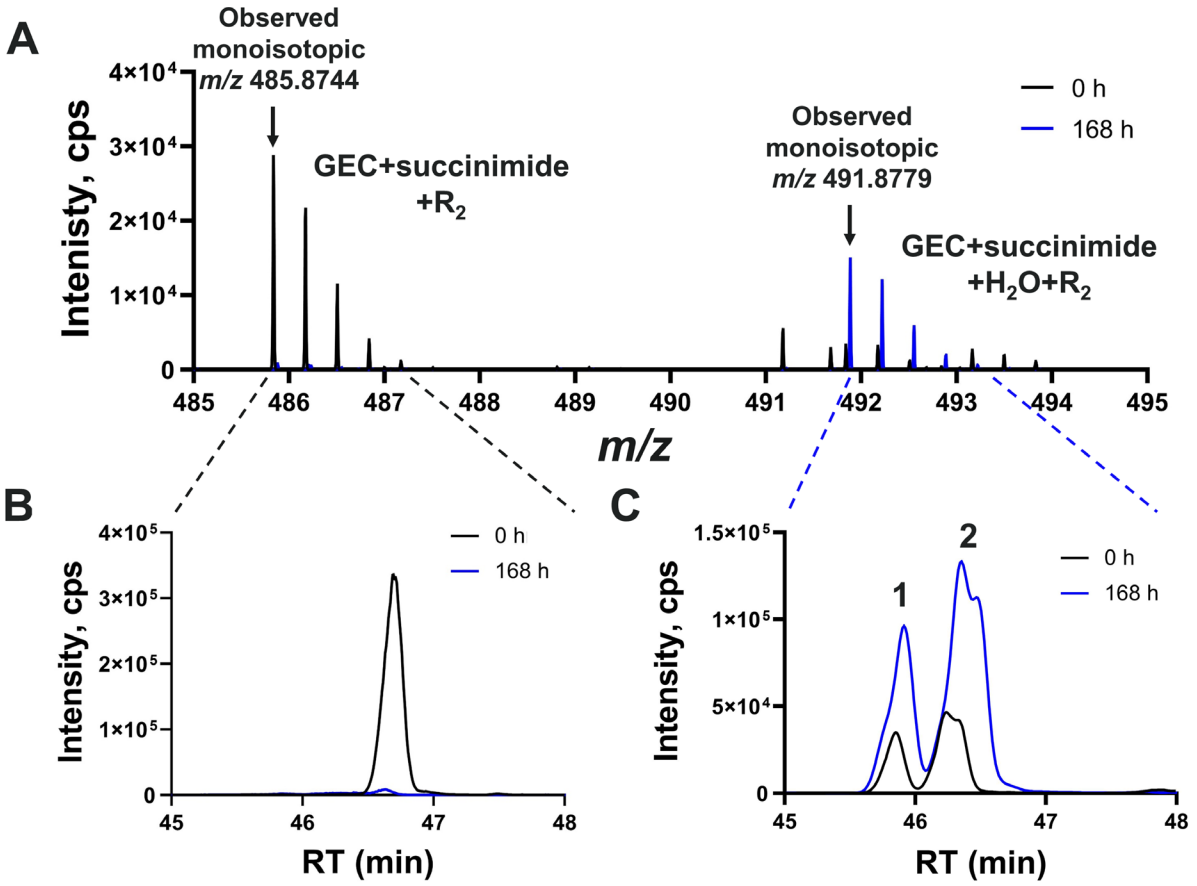

Figure 2. (A) Overlaid mass spectra of GEC+succinimide+R_2 and GEC+succinimide+H_2O+R_2 from 0 h (black line) and 168 h (blue line) plasma incubated ADC1 samples. Both species observed a charge state of 3. Overlaid XICs of (B) GEC+succinimide+R_2 or (C) GEC+succinimide+H_2O+R_2. Black line represents the sample with no incubation, whereas the blue line is the sample incubated for 168 h.

3.2 Identification of thio-succinimide hydrolysis isomeric products

The hydrolysis of thio-succinimide creates a pair of constitutional isomeric products, as shown in Figure 2A and reported previously. In Figure 2C, two distinctive peaks with different retention times observed in XIC confirm the presence of GEC+succinimide+H_2O+R_2 isomers. The peak

with RT = 45.9 minute is denoted as peak 1, whereas the peak with RT = 46.4 minute is denoted as peak 2. In order to elucidate the chemical structure corresponding to each peak, we employed two orthogonal fragmentation methods, CID and EAD, on GEC+succinimide+H_2O+R_2. Figures S2A-B show the CID MS/MS spectra of precursor ion from peak 1 and 2, respectively. However, we did not find any distinctive peaks by comparing these two spectra. Hence, CID does not provide useful information in distinguishing thio-succinimide hydrolysis isomers.

Differentiation between aspartic acid and iso-aspartic acid from asparagine deamidation has been previously reported using electron-involved dissociation (ExD) fragmentation methods, such as electron-transfer dissociation (ETD) and electron-capture dissociation (ECD). c+57 and z-57 were confirmed to be a pair of diagnostic ions to identify iso-aspartic acid generated from either ETD or ECD fragmentation.[26, 28] Asparagine deamidation first generates a succinimide intermediate prior to ring-opening hydrolysis, which is analogous to thio-succinimide hydrolysis as shown in Figure 2A. Figure S3 shows the mechanism of asparagine deamidation and structures of diagnostic c- and z-related ions. Figure S4 shows the fragmentation sites, and the structures of c- and z-related ions in both deamidation and thio-succinimide hydrolysis isomers. Figures 3A and 3B are the MS/MS spectra (*m/z* range: 100-2900) of GEC+succinimide+H_2O+R_2 precursor ions from peak 1 and 2, respectively, using EAD fragmentation. The product ions in both figures show high agreement with each other. (I), (II) and (III) in Figures 3A and 3B are zoomed-in MS/MS spectra with different *m/z* range. Comparing Figures 3A (I) and 3B (I), we found a singly charged fragment ion with monoisotopic *m/z* of 365.0839, which is in accordance with R_1+Thio+57 diagnostic ion with a -15 ppm mass difference. Similarly, by comparing Figures 3A (III) and 3B (III), we found a singly charged fragment ion with monoisotopic *m/z* of 1109.5403. This peak suggests the presence of R_2+Succ+H_2O-57 diagnostic ion with -12 ppm mass difference. Thus, we can confirm Figure 3A represents the MS/MS spectrum of thio-Asp from peak 1, whereas Figure 3B is the MS/MS spectrum of thio-isoAsp from peak 2. Observed *m/z* values of discussed ions all show similar extent of deviation to their theoretical values. A recalibration performed after acquisition corrects the *m/z* difference to within ±3 ppm (see Section S3 in the supporting information). Hence, the identification of ions of interest is accurate, despite the mass errors being close to the high end of tolerance.

Another notable difference is R_2+Succ+H_2O-44 (indicated by pink arrow) shows significantly higher intensity in Figure 3A, whereas the intensity of the same fragment ion in Figure 3B is low. Figures S5A and S5B show zoomed-in MS/MS spectra from both peak 1 and 2 at *m/z* 555-570 range, respectively. The higher signal intensity of R_2+ Succ+H_2O-44 in Figure S5A suggests

the CO_2 loss after forming R_2+ Succ+H_2O ion of thio-Asp is significantly enhanced. This finding aligns with the ExD fragmentation results of aspartic acid in deamidation.

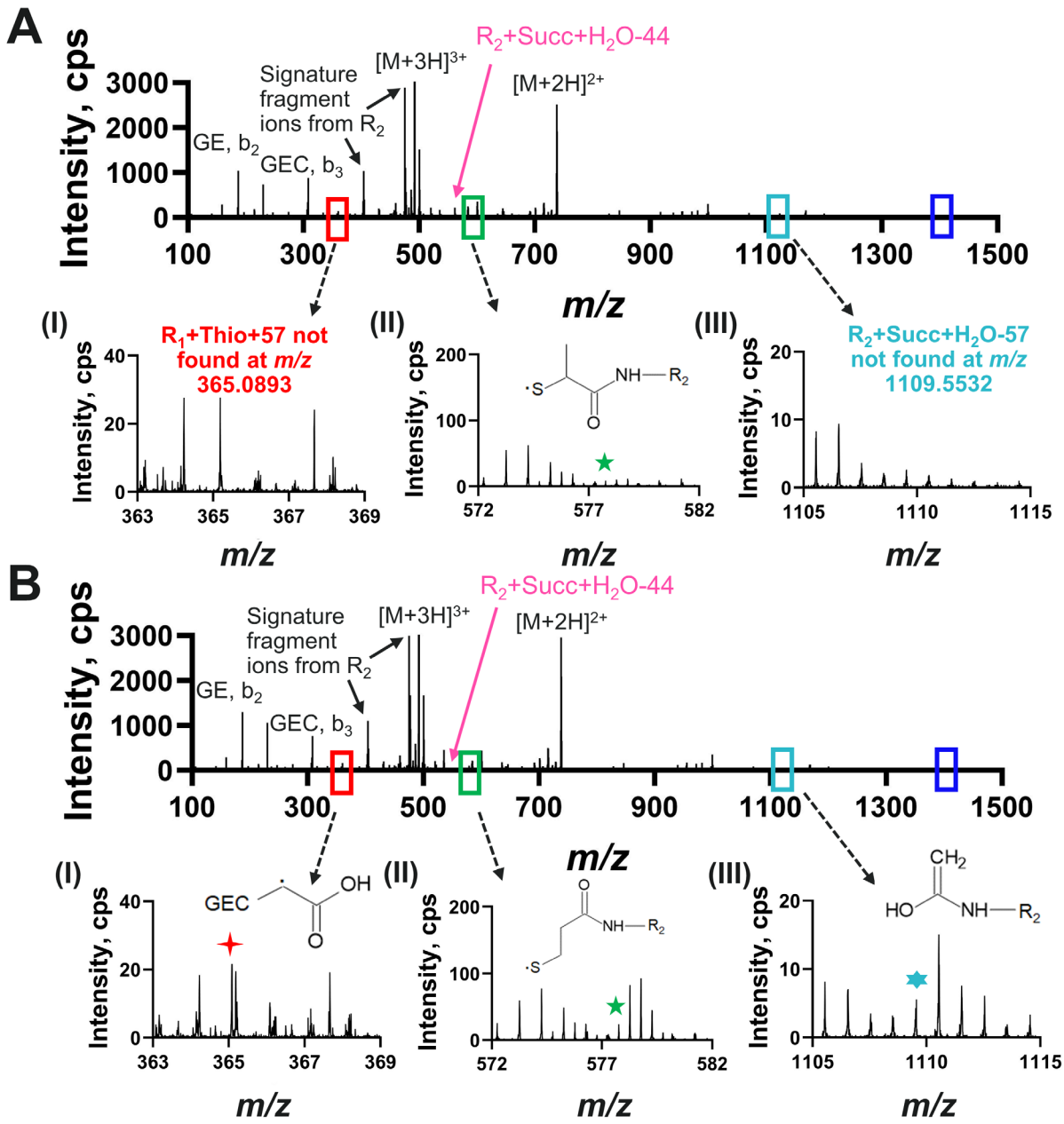

Figure 3. EAD MS/MS spectra (*m/z* 100 to 1500) of (A) peak 1 and (B) peak 2 observed in Figure 2C. Signature fragment ions are labelled on both spectra. Pink arrows point to a diagnostic ion, which has a structure of R_2+Succ+H_2O-44. Dashed arrows point to zoomed-in EAD MS/MS spectra with different *m/z* ranges. (I) *m/z* 363-369, (II) *m/z* 572-582, and (III) *m/z* 1105-1115. Blue boxes are the zoomed-in spectra of *m/z* 1410-1440 (see Figure S7). The stars with 4, 5 and 6 arms in inset figures correspond to the monoisotopic peak of each diagnostic fragment ions, respectively. Their observed *m/z* values are 365.0839, 577.7643 and 1109.5403, respectively. R_2+Succ+H_2O-44 ion was also observed in Figure 3A (II) with -12 ppm difference to its theoretical value, although the intensity is low.

We also observed a doubly-charged fragment ion with a monoisotopic *m/z* of 561.2728 with enhanced intensity in Figure 3A (II), but low intensity in Figure 3B (II). We propose the structure of thio+succinimide+H_2O+R_2-CO_2 (Figure 1). We denote this fragment ion as R_2+ThioSucc+H_2O-44. Although both thio-Asp and thio-isoAsp are capable of CO_2 loss after forming the R_2+ThioSucc+H_2O fragment ion, clearly, thio-isoAsp is more prone to this neutral loss. We also found the R_2+ThioSucc+H_2O fragment ion in both thio-Asp and thio-isoAsp with similar intensities, as shown in Figure S6A and Figure S6B. Thus, R_2+ThioSucc+H_2O-44 fragment ion can serve as an additional diagnostic ion for differentiating thio-Asp and thio-isoAsp. To our knowledge, this fragment ion has never been reported in the context of differentiation of deamidation isomers. Hence, this diagnostic ion is distinctive of the thio-succinimide system. We infer that the generation of this unique fragment ion is related to the thiol group linked with the succinimide ring and R_2.

Neutral losses are not limited to the fragment ions generated from ExD fragmentation. M-60 was reported to be a diagnostic ion for Asp in deamidation.[29] Figures S7A and S7B show the zoomed-in MS/MS spectra of Figures 3A and 3B at *m/z* 1410-1440. To our surprise, the M-60 fragment ions (observed monoisotopic *m/z* 1414.6355, 10 ppm difference to its theoretical value) in both Figures S7A and S7B show similar intensity. Thus, M-60 cannot differentiate thio-Asp and thio-isoAsp, and we think the 60 Da neutral loss might come from the contribution of R_2. We also observed peaks with monoisotopic *m/z* 1430.6428 (-7 ppm difference to theoretical *m/z* of M-44) in both isomers. The similar intensities of M-44 and M-60 suggest that neutral losses to the whole precursor ion cannot distinguish thio-Asp and thio-isoAsp.

Furthermore, we also investigated another tryptic peptide SCDK that contains succinimide+R_2 from ADC1 followed by the same sample preparation steps. The SCDK+succinimide+R_2 is mostly hydrolyzed after 168 h of incubation, as shown in Figure S8A. Both XICs of non-hydrolyzed and hydrolyzed at 0 and 168 h in Figures S8B and S8C support the same conclusion. Additionally, Figure S8C shows two distinct peaks that were well separated under current LC gradient. The presence of two peaks suggests the formation of isomers after thio-succinimide hydrolysis. This finding aligns with our observations of GEC-linked succinimide structure.

4. CONCLUSION

The formation of thio-succinimide hydrolysis isomeric products is well known, however, the differentiation between them has not been sufficiently studied so far. This work demonstrated a reference material-free characterization of hydrolyzed thio-succinimide isomers using EAD fragmentation. Similar to the differentiation between aspartic acid and iso-aspartic acid generated from asparagine deamidation, we observed distinctive R_1+Thio+57, R_2+Succ+H_2O-57 for thio-isoAsp and enhanced R_2+Succ+H_2O-44 for thio-Asp in EAD MS/MS spectra. However, M-44 and M-60 failed to serve as diagnostic ions due to their similar intensities in EAD MS/MS spectra for both thio-Asp and thio-isoAsp. To our surprise, we observed a significantly enhanced CO_2 neutral loss after formation of the R_2+ThioSucc+H_2O fragment ion for thio-isoAsp. This fragment ion is denoted as R_2+ThioSucc+H_2O-44, and for the very first time, we report it as a

unique diagnostic ion for differentiating thio-Asp and thio-isoAsp. To our knowledge, a similar fragment ion has not been identified for asparagine deamidation. The formation of this fragment ion might relate to the thiol group linked to succinimide ring and the structures of R_2. For instance, in the case of deamidation, the succinimide ring is linked with amino acids at both ends. Compared to the conventional approach which matches RT of purified reference material to distinguish isomers, EAD fragmentation serves as a novel tool to identify isomeric structures without reference standards. The differentiation between thio-Asp and thio-isoAsp may eventually benefit the drug development where thiol-maleimide conjugation is employed.

AUTHOR CONTRIBUTIONS

Junyan Yang**:** Conceptualization; methodology; investigation; formal analysis; writing-original draft; visualization. Jiaqi Yuan: Conceptualization; methodology; investigation; draft review. Yue Huang: conceptualization, methodology, draft review. Anton I. Rosenbaum: conceptualization; methodology; investigation; draft review, supervision.

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CONFLICT OF INTERESTE STATEMENT

J. Yang, J. Yuan, Y. H. and A. I. R. are or were employees of AstraZeneca at the time this work was conducted and may hold stock and/or stock options or interests in the company.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.